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Cutting Edge: Diminished T Cell TLR Expression and Function Modulates the Immune Response in Human Filarial Infection

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Patent lymphatic filariasis is characterized by profound Ag-specific T cell hyporesponsiveness with impaired IFN-γ and IL-2 production. Because T cells have been shown to express a number of TLR and to respond to TLR ligands, we hypothesized that diminished T cell TLR function could partially account for the T cell hyporesponsiveness in filariasis. T cells expressed TLR1, TLR2, TLR4, and TLR9, and the baseline expression of TLR1, TLR2, and TLR4, but not TLR9 was significantly lower in T cells of the filarial-infected individuals compared with the uninfected individuals (both endemic and nonendemic). TLR function was significantly diminished in the T cells of filarial-infected individuals based on decreased T cell activation/cytokine production in response to TLR ligands. Thus, diminished expression and function of T cell TLR is a novel mechanism underlying T cell immune tolerance in lymphatic filariasis. The Journal of Immunology, 2006, 176: 3885–3889.

Impairment of Ag-driven Th1 responses is characteristic of lymphatic filarial infection (1). One of the major mechanisms underlying the impaired immune responses is dysregulated APC function (2). We have previously determined that spontaneous and/or parasite Ag-induced expression of TLR1, TLR2, TLR4, and TLR9 is diminished in B cells and monocytes of filarial-infected individuals (3), and that TLR ligand-stimulated activation and cytokine induction is impaired in B cells and monocytes from filarial-infected individuals (3).

Recently, reports have postulated a role for T cell TLR signaling in influencing adaptive immune responses. At the mRNA level, human T cells have been shown to express several TLR, suggesting that TLR ligands could directly influence T cell function (4, 5). In addition, expression and function of TLR2 and TLR4 (6) as well as TLR5 and TLR7/8 (7) has been demonstrated on highly purified activated/memory T cells. In these systems, TLR function as costimulatory receptors, amplifying the immune response in concert with TCR-dependent stimuli. In addition, murine T cells have been shown to express TLR4 (8) and TLR9 (9), with TLR4 and TLR9 ligands inducing an activation/proliferation response.

To elucidate the role of T cell TLR in filarial infections, we examined expression of TLR1, TLR2, TLR4, and TLR9 in a cohort of 10 infected individuals (INF) and 10 uninfected individuals (UN), along with 10 nonendemic North American normal blood donors (BB). Using flow cytometry, we demonstrate T cell expression of TLR1, TLR2, TLR4, and TLR9. In addition, patent filarial infection is associated with diminished expression of TLR1, TLR2, and TLR4, but not TLR9, which has functional consequences in terms of TLR-mediated responses. Moreover, there was a direct effect of TLR ligands on T cells (in the absence of APC), suggesting an important role for TLR expression on T cells and their function in chronic filarial infections.

Materials and Methods

Study population

We studied a cohort of 20 individuals in an area endemic for lymphatic filariasis in Tamil Nadu, South India. This included 10 clinically asymptomatic, infected individuals (INF), and 10 UN, and their clinical and parasitological profile has been described previously (3). BB at the National Institutes of Health were used as nonendemic controls, because their serum tested negative for filarial Ag.

Reagents

Ab used for phenotype analysis were obtained from BD Pharmingen/BD Biosciences, and Ab used for TLR analysis were obtained from ebioscience. The Abs used in the study were as follows: FITC-labeled anti-human CD56; PE-labeled anti-human TLR1, TLR2, TLR3, TLR4, TLR9, CD69, CD25, IFN-γ, and TNF-α; and PerCP-labeled anti-human CD3. The TLR ligands (InvivoGen) used were as follows: TLR2 ligand, Pam3CysSerLeu4 (hereafter Pam3Cys); TLR3 ligand, poly(I:C); TLR4 ligand, ultrapure LPS; and TLR9 ligand, CpG DNA M362 (hereafter CpG).

Isolation of PBMC and in vitro culture and cell purification

PBMC were isolated as described previously (10). PBMC were cultured in 24-well tissue culture plates (Corning) at concentrations of 5 × 10⁶/well with Pam3Cys (10 μg/ml), ultrapure LPS (10 μg/ml), or CpG DNA (5 μM) for 24, 48, and 72 h. For purified T cell stimulation, T cells from BB were purified by
negative selection using the T cell selection kit (Miltenyi Biotec) magnetic column separation. The purity of T cells was between 97 and 99% in each of our isolations. T cells were then cultured with anti-CD3 (5 μg/ml) and Pam3Cys (10 μg/ml) or ultrapure LPS (10 μg/ml), CpG DNA (10 μg/ml), or poly(i:C) (50 μg/ml) (all obtained from InvivoGen) for 24, 48, or 72 h.

Flow cytometry
PBMC were washed in PBS/0.1% BSA buffer, and staining was done using a permeabilization buffer (PBS/0.1% BSA/0.1% saponin) for detection of intracellular expression. Fluorescence was measured on a FACSCaliber (BD Biosciences) using 50,000-gated lymphocytes.

Statistical analysis
Comparisons between groups were done using the nonparametric Mann-Whitney test. All statistics were performed with StatView 5 software (SAS Institute). Results

T cells express TLR1, TLR2, TLR4, and TLR9
We examined the expression of TLR1, TLR2, TLR4, and TLR9 on T cells from different groups of individuals—INF, UN, and BB by flow cytometry. We gated on CD3+CD56- T cells to exclude NK and NKT cells. A representative dot plot is shown in Fig. 1, indicating the presence of T cells expressing TLR1, TLR2, TLR4, and TLR9. TLR9 expression was determined by both surface and intracellular staining, and almost all of the staining was detected intracellularly. Detectable levels of TLR1, TLR2, and TLR4 expression was found in T cells from UN and BB and, for TLR9, in T cells from all groups (UN, INF, and BB). The expression of TLR9 was detected on activated/memory T cells (CD45RO+) of both the CD4+ and CD8+ T cell compartment but not naive T cells (CD45RA+) or regulatory T cells (CD4+, CD25+) (data not shown). As observed previously (3), non-CD3+ lymphocytes, mainly B cells, also express TLR9, and the expression is significantly lower in INF.

Figure 1. T cell expression of TLR1, TLR2, TLR4, and TLR9. PBMC from a representative filarial-infected (INF), uninfected (UN), and nonendemic normal (BB) individual were stained for surface TLR1, TLR2, TLR4, and intracellular TLR9. The quadrants for each sample were drawn on the basis of isotype control Abs for each TLR. Data are shown as a dot plot with the x-axis representing CD3+CD56- T cells and the y-axis the respective TLR. The numbers indicate the percentage of CD3+ T cells positive for each TLR.

Decreased expression of TLR1, TLR2, and TLR4 in INF
To estimate the expression levels of T cell TLR in INF (n = 10), UN (n = 10), and BB (n = 10), we compared baseline expression of TLR on CD3+CD56- cells. The percentage of T cells expressing TLR1 in INF (GM = 0.057) was significantly lower compared with UN (GM = 1.497; p = 0.047) and BB (GM = 0.826; p = 0.021) (Fig. 2). Similarly, the percentage of T cells expressing TLR2 in INF (GM = 0.023) was significantly lower compared with UN (GM = 1.64; p = 0.029) and BB (GM = 1.41; p = 0.018). Also, the percentage of T cells expressing TLR4 was significantly lower in INF (GM = 0.326) compared with UN (GM = 1.520; p = 0.025) and BB (GM = 0.927; p = 0.108); however, the expression of TLR9 was not significantly different among the three groups (INF, GM = 4.58; UN, GM = 7.29; and BB, GM = 5.27) (Fig. 2). The geometric mean fluorescence intensity of TLR1 (38 in INF vs 61 in UN and 65 in BB), TLR2 (42 vs 68 and 75), and TLR4 (37 vs 61 and 59), but not TLR9 (452 vs 454 and 462) was significantly lower in T cells of INF compared with both UN and BB (data not shown). TLR3 expression was not detected in T cells. These data indicate that both the percentage of T cells expressing TLR1 and the density of TLR expression per T cell is diminished in INF.

T cell responses to a TLR2 ligand are diminished in INF
To determine whether the altered TLR expression in T cells of INF translated to functional diminution in responses to TLR ligands, we stimulated PBMC with Pam3Cys, LPS, CpG, and, as a control, poly(I:C) for 24, 48, or 72 h, and examined the T cell response by flow cytometry in INF (n = 5) and UN (n = 5) (Fig. 3). Functional responses were observed at 72 h following stimulation. Although baseline expression of all markers and cytokines examined was not different between the two groups (data not shown), Pam3Cys induced a significant up-regulation of CD69 (fold change GM of 3.30 in UN vs 1.21 in INF; p = 0.0209) and CD25 (4.11 vs 1.53; p = 0.023) in INF compared with UN. When T cell cytokine production was examined by intracellular flow cytometry, we found that Pam3Cys increased the percentage of T cells expressing IFN-γ (percent GM of 4.41 in UN vs 1.92 in INF; p = 0.0433) and TNF-α...
(3.34 vs 1.35; \( p = 0.0209 \)) in UN compared with INF. Although TLR4 expression was lower in INF, LPS did not induce a lower activation of INF T cells, nor did CpG. Poly(I:C) did not alter the expression of any of the activation markers or nature of cytokine secretion, suggesting the functional responses measured correlated with the expression patterns of TLR in INF and UN.

Having established a relationship between diminished T cell TLR function to infection status, we next attempted to differentiate between two underlying mechanisms in which either 1) TLR ligands act on APC with subsequent APC-mediated activation of T cells or 2) TLR ligands could interact directly with T cells to induce activation. Uninfected cells were isolated and exposed to TLR ligands, and the expression of activation markers was measured.

**FIGURE 2.** Diminished expression of TLR on T cells in patent lymphatic filariasis. The percentage of CD3+ T cells expressing TLR1, TLR2, TLR4, and TLR9 in filarial-infected (INF), uninfected (UN), and nonendemic normal (BB) individuals \(( n = 10)\) in each group. Values of \( p \) were calculated using the Mann-Whitney test.

**FIGURE 3.** Stimulation of T cells by TLR ligands. Fold change over baseline of CD69, CD25, IFN-\( \gamma \), and TNF-\( \alpha \) in T cells of UN \(( n = 5)\) and INF \(( n = 5)\) in response to ligands for TLR2 (Pam3Cys), TLR4 (LPS), and TLR9 (CpG) is expressed as box plots, with the horizontal lines representing the 25, 50, and 75th percentiles of the data. Values of \( p \) were calculated using the Mann-Whitney test.

Purified T cells express IFN-\( \gamma \) in response to TLR ligand in a TCR-dependent manner.
with T cells to initiate a response. To distinguish between the two mechanisms, we purified T cells from BB and stimulated them with Pam3Cys, poly(I:C), LPS, and CpG for 24, 48, or 72 h. We did not observe any activation of purified T cells cultured with TLR ligands alone at any time point. Because TLR ligands have been postulated to act as costimulators of T cell activation with anti-CD3, we cultured purified T cells with anti-CD3 and individual TLR ligands and observed functional responses at 48 h. As shown in a representative histogram in Figs. 4A, the percentage of CD3⁺CD56⁻ T cells expressing activation markers CD69, CD25, and IFN-γ was increased in response to Pam3Cys, LPS, and CpG in concert with anti-CD3; however, on statistical analysis, T cell expression of IFN-γ was significantly increased only in response to anti-CD3 plus Pam3Cys in comparison to anti-CD3 alone (p = 0.0374) or anti-CD3 plus poly(I:C) (p = 0.0163) in BB (n = 5) (Fig. 4B). Thus, TLR2 expression of T cells results in a direct functional interaction of activated T cells with TLR2 ligands.

**Discussion**

The induction of host evasion and regulatory mechanisms plays a vital role in establishing the chronicity of filarial infections (11). Filarial parasite modulation of activation, survival, and cytokine responses of APC is now well established (3). Similarly, the expression and function of the TLR family of pattern-recognition receptors, crucial in sensing and responding to infectious and inflammatory cues, is impaired in APC of chronically

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**FIGURE 4.** TLR ligand stimulation of purified T cells. A. A representative histogram showing the percentage of purified T cells from BB expressing CD69, CD25, or IFN-γ following 48-h stimulation with anti-CD3 and Pam3Cys, LPS, CpG, or poly(I:C). B, Fold change over baseline of CD69, CD25, and IFN-γ in T cells of UN (n = 5) and INF (n = 5) in response to ligands for TLR2 (Pam3Cys), TLR4 (LPS), and TLR9 (CpG) is expressed as box plots, with the horizontal lines representing the 25, 50, and 75th percentiles and the vertical lines representing the 10 and 90th percentiles of the data. Values of p were calculated using the Mann-Whitney test.
infected, microfilaremic but asymptomatic filarial patients. Although effects mediated by filarial parasites on APC have been well documented, very little is known about the direct effect of filarial parasites on T cells.

Expression of TLR on T cells has recently been characterized at the mRNA and protein levels. Thus, in humans, expression of TLR2 and TLR4 on the surface of T cells with TLR2 functioning as a costimulatory receptor on activated T cells has been demonstrated (6). Similarly, the expression of TLR5, TLR7, and TLR8 at the mRNA level as well as functional activity attributed to TLR5 and TLR7/8 ligands in a TCR-dependent (anti-CD3) or TCR-independent (anti-CD2 or IL-2) manner has also been shown (7). T cells in mice have been reported to express TLR4 and TLR9, which can respond to LPS and CpG DNA (8, 9). In this study, we have performed a comprehensive examination of T cell expression of different TLR. T cells were found to express low but consistently reproducible levels of surface TLR1, TLR2, TLR4, and intracellular TLR9 in three different groups of individuals: INF, UN, and BB. In addition, we observed significantly diminished expression of TLR1, TLR2, and TLR4 on T cells in INF compared with UN and BB. Notably, no difference in the expression patterns of TLR9 was noted on T cells obtained from peripheral blood of the three different groups. Because a down-regulation of TLR expression is an important immune evasion strategy used by different bacterial and protozoan pathogens (12), this study suggests that parasite Ag-specific down-regulation of TLR1 and TLR2 may be another mechanism that helminth parasites use to directly dampen T cell responses that are detrimental to their development.

The T cell TLR function studies demonstrated that T cell activation markers (CD69 and CD25) and Th1 cytokines (IFN-γ and TNF-α) in response to Pam3Cys are significantly diminished in INF compared with UN. Thus, expression patterns of TLR on T cells directly translate into functional differences and indicate a novel mechanism of down-regulated Th1 responses in filarial infections, that being a direct effect on effector T cells through parasite-induced TLR down-modulation. Notably, T cell responses were first observed 72 h following exposure, unlike APC responses, which have been shown to occur much earlier (3).

The T cell TLR responses to the respective TLR ligands occurred in the presence of accessory cells (B cells and monocytes) because total PBMC from INF and UN was used in the culture system. To determine whether T cells respond to TLR ligands in the absence of APC, we purified T cells from BB and stimulated them with TLR ligands. In the absence of any other stimulus, we did not observe any functional responses even at 72 h following exposure. This indicates that T cells do not respond directly to TLR ligands in the absence of any other stimuli/ accessory cells. The lack of direct activation of T cells by TLR ligands probably reflects a control mechanism to ensure absence of self-reactivity and autoimmunity; however, other studies have shown that activated T cells can respond to TLR ligands in the presence of a costimulus (6, 7), and indeed, in our study, we observed activation of T cells in the presence of anti-CD3 by Pam3Cys. No significant activation of T cells by LPS and CpG DNA occurred, confirming previous observations that human T cells do not respond to all TLR ligands promiscuously. Moreover, no alteration in TLR expression levels was observed following T cell activation.

TLR2 is a receptor that plays an important role in filarial infection. Thus, the filarial endosymbiont Wolbachia is known to elicit immune responses through TLR2 and TLR4 and is known to be the major mediator of inflammatory responses in lymphatic filariasis and onchocerciasis (13, 14). Although monocytes were previously thought to be the major source of inflammatory responses in filarial infections (14), it is now clear that T cells may also play a significant role (10). Thus, we propose that TLR2 expression on Ag-specific activated/memory T cells could facilitate interaction of filarial Ags with the adaptive immune system of the host. This interaction could be an important mechanism of host inflammatory responses to different stages of the filarial parasites. Although TLR2 expression levels are low in T cells, TLR2 expression clearly has functional significance as reflected by the increased activation in response to Pam3Cys plus anti-CD3, indicating that TLR2 is an important costimulatory molecule on T cells. By down-modulating the expression of TLR2, filarial parasites could manipulate the host environment to evade hostile proinflammatory responses and establish a milieu for long-standing, chronic infections. Our studies suggest a central role for TLR dysregulation as a mechanism for induction and maintenance of Ag-specific anergy in filariasis and implicate TLR2 down-regulation on T cells as a direct mechanism of diminished Th1 responses.

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Disclosures
The authors have no financial conflict of interest.

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